STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

NO FURTHER CERTIFICATION OF GENTIAN VIOLET

Recently the following announcement has been sent to the American stain manufacturers who have been submitting their products to the Stain Commission for Certification:

"Beginning Jan. 1, 1942, we will no longer certify any dye under the name 'gentian violet'. It must be labelled 'crystal violet', 'methyl violet 2B', or whatever it may be. After this name may be added a statement: 'Formerly sold as gentian violet', but this statement must be in small type."

Possibly a few words of explanation should be added to this statement for the benefit of those who have in the past been using one of these dyes under the name "gentian violet". This name has never had any very definite meaning, and has never been recognized in dye indexes. Biologists, however, have always been familiar with the term, as Dr. Grübler, who first attempted to standardize biological stains, continuously sold a mixture of dyes under this name. His "gentian violet" was stated to be a mixture of crystal violet and methyl violet diluted with 50% dextrin.

This product may have been quite constant in composition as long as Dr. Grübler was in charge of its preparation; but it proved very difficult for any other manufacturer to duplicate. The reason for this is clear. Altho crystal violet is a definite chemical compound (hexa-methyl pararosanilin), methyl violet is a variable mixture often containing this same hexa-methyl pararosanilin together with the penta-methyl and tetra-methyl homologs. This relationship has been repeatedly discussed in the pages of this journal, and for more detailed information the reader is referred to Biological Stains.[1] Enough here to state that a mixture of methyl violet and crystal violet would presumably be identical with one of the bluer shades of methyl violet, as for instance methyl violet 2B, 3B or 4B.

When the work of standardizing biological stains was begun in this

[1]Conn, H. J. 1940. Biological Stains. Biotech Publications, Geneva, N. Y. *4th Ed.* See pp. 122–6.

country, it was at first desired to eliminate the name "gentian violet" in favor of one or two of the more definite names listed in dye indexes. This suggestion, however, met with considerable opposition both on the part of users and dealers of biological stains; the former were always used to ordering "gentian violet" and the latter wanted to know how they could supply the demand if not allowed to sell a dye under that name. Finally, therefore, the Stain Commission defined "gentian violet" in the 1st edition of Biological Stains (p. 69) as "either penta-methyl or hexa-methyl pararosanilin or else a mixture of methylated pararosanilins composed primarily of the two compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B".

This has never been recognized as a satisfactory solution of the question, and in the last edition of Biological Stains the following statement is made: "The Commission does not wish to continue to give official recognition to a dye of such indefinite nature as gentian violet; and as soon as users of stains seem to be sufficiently educated in the matter, certification will be refused on any dye of this name." This time now seems to have come; accordingly the announcement to the stain companies quoted above.

Many users of stains have for some time been ordering either crystal violet or methyl violet instead of "gentian violet". For the benefit of those still using one or the other out of bottles bearing the last-mentioned label, the following information is given to guide them in what to use in the future: The National Aniline Co. and the Hartman-Leddon Co. have both been selling the same dye as either crystal violet or "gentian violet" for at least 15 years; Coleman and Bell have been selling one of the bluer methyl violets under the name "gentian violet, improved", and the same product will undoubtedly be available from them in the future labelled more definitely but with a statement added as to the name under which it was formerly sold; the European "gentian violets" when last tested were methyl violets, but none of them have been recently analyzed. This information will help a user in duplicating a batch of "gentian violet" already on hand; if further information is needed the following hint from the last edition of Biological Stains may be useful: "Users should specify crystal violet for bacteriological work, and for histological work where a deep blue-violet is required; but should order methyl violet 2B in histological procedures where a reddish shade is called for."—H. J. Conn.

PLANT-VIRUS DIFFERENTIATION BY TRYPAN-BLUE REACTIONS WITHIN INFECTED TISSUE[1]

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ABSTRACT.—**Trypan blue has proved effective for demonstrating the presence of certain plant viruses within infected tissues. The amorphous and crystalline inclusions which constitute cytological evidence of viruses stain proportionately. The effects produced by different viruses react differently to the stain and those inclusions which do not absorb trypan blue tend to stain with phloxine. This selective staining is the basis for using trypan blue singly and in combination with phloxine as standardized procedures for demonstrating and differentiating cytological evidence of plant viruses. These tests are very rapid and are especially applicable to temporary mounts of living tissue but permanent mounts can be made from material fixed in formalin.**

It has long been known that the cells of some plant species, when infected with certain viruses, contain cell inclusions which are not found in uninfected plants of the same species. These inclusions are of many types varying from crystalline or para-crystalline structures to amorphous masses. The amorphous inclusions have been called x-bodies. These various inclusions are protein in nature and are, in part at least, the virus itself (Bawden, 1939). It is not surprising, therefore, that cytological changes wrought by viruses are often just as definite and sometimes more definite than the gross morphological changes by which plant-virus diseases are usually identified. Inclusions are therefore referred to in this paper as "virus evidence".

Better methods have long been needed for investigating cytological evidence. Paraffin methods are very unsatisfactory because the process may change the relatively unstable products of virus activity and because sections seldom permit one to visualize the cell changes as a whole and relate the virus effects to the norm. In fact a critical survey of the literature indicates that examination of paraffin sections is directly responsible for the conflicting statements in literature

[1]Published as Technical Paper No. 371 with the approval of the Director, Oregon Agricultural Experiment Station. Contribution of the Department of Botany in Cooperation with the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

regarding the occurrence and nature of x-bodies. Whole mounts are far more instructive than thin sections except for special cases such as phloem studies. Dr. Sheffield has combined the Feulgen reaction with the dioxan whole-mount method. This process is effective for nuclear differentiation but it does not permit proper analysis of the inclusions. Examination of living infected material with visible and ultra violet light, and frequent comparison with non-infected material is essential for a reliable interpretation of virus evidence. With this viewpoint in mind the writer has tested many vital and semivital stains to find one which would abet examination of living material and also be applicable to material fixed in neutral formalin. Trypan blue (N.A.Co. 7286) has been found specific for this purpose. The dye has an affinity for the crystalline virus elements of some inclusions and for nuclei. This coincident staining is significant since Bawden (1939) and others have shown plant viruses are chemically related to nucleoproteins. On the other hand, some viruses are associated with inclusions which are not readily stained by trypan blue. The reaction, therefore, may be used as a differential test. This paper gives directions for its use first (Test 1) for those viruses whose inclusions are readily stained and secondly (Test 2) in combination with phloxine for those viruses whose inclusions are not stained by trypan blue alone.

The application of these methods to various plant viruses has already justified the use of the term "viroplast" for inclusions formerly classified as "amorphous" or "x-bodies" since it has been possible to show there are at least ten distinct forms of these, some of which contain demonstrable crystalline elements (McWhorter, 1940). Unfortunately, these tests may affect the protoplasts and induce plasmolysis and other changes which must be constantly checked with living material before one can form a final opinion about the constitution of inclusions or other cytological evidence, even tho these may be vividly shown by the stains.

Stains and Reagents. The stains required are 0.5% and 0.05% trypan blue and 0.5% phloxine, all made up in physiological salt solution (0.85% aqueous NaCl). The effectiveness of these staining reactions depends on using a suitable detergent or wetting agent to avoid air bubbles in tissue mounts and insure uniform penetration of stains. A 1% solution of the chemically pure form of Vatsol known as O. T. 100, dissolved in hot water, has proved excellent for these and many other laboratory technics.

Preparation of Plant Material for Mounts. The surface of leaves or stems should be gently rubbed with water, or if they are difficult

to wet, they should be dipped in the detergent for about 10 seconds, then gently rubbed in water. Peelings should be made by cutting into the tissues parallel to the surface and then stripping off the piece. This can be done easily with a thin chisel knife made of razor steel which can be sharpened so that the edge will slip under the epidermis of even a thin leaf. The peelings should be immersed in the detergent for not more than 3 seconds, and then passed into a dish of physiological salt solution. Duplicate peelings should be available so that the two following tests can be started simultaneously.

Test 1—Trypan Blue Absorption. Pour 0.05% trypan blue into a watch glass and place one or more peelings in the liquid.—If virus evidence reacts positively to trypan blue, the peelings will assume a light blue tone in 15 minutes. The end-point in the staining is reached in 30 minutes. Nuclei assume a light blue color and virus evidence becomes intensely blue. Other parts of living cells do not stain appreciably, but certain tissues such as xylem will absorb the stain strongly. If the subject reacts negatively to this dye, very little color will be absorbed by living cells in 30 minutes, since only the nuclei stain. Chloroplasts remain green, a circumstance that enables one to examine readily the chlorenchyma cells in the cut ends of the peelings, since blue stained inclusions, especially crystals, contrast with the green (Plate 1, Fig. 2). Starch grains, elaioplasts, and oil globules are not stained.

It should be noted that nuclei in healthy or diseased tissue swell noticeably during the staining process. This can be circumvented by first fixing in formalin as outlined below.

Test 2—Phloxine-Trypan-Blue. Immerse the peeling in 0.5% phloxine for 3 to 8 seconds. Remove quickly and wash briskly in physiological salt solution. Transfer the peeling, which should now appear pink, into a few drops of the 0.5% trypan blue, for at least 2 to 4 minutes unless the piece of tissue is very small. Rinse and mount in physiological salt solution.—The viroplasts that do not absorb trypan blue should now appear bright pink or even dark red. In locations where the stains balance properly, the nuclei will be deep blue and the viroplasts pink or purplish, according to their ability to absorb trypan blue. Some crystalline inclusions remain unstained. Chloroplasts remain green and virus evidence becomes conspicuous even in chlorenchymatous cells. The effect is not unlike a Giemsa blood stain except that chlorophyll adds a green coloration to the array. The chief purpose of Test 2 is to stain differentially and to make readily discernible those viroplasts that do not absorb trypan blue when used as directed in Test 1.

Special Cases and Variations. Material which cannot be examined at once may be fixed in 5% formalin. As fixation progresses, the staining reactions become less and less effective. Protein cell contents become yellow in formalin and are thereby made more visible, but this coloration is very unsatisfactory for direct examination or photographic rendering. It therefore seemed desirable to the author to treat the fixed material with some reagent that would restore the staining properties of the tissue. At the suggestion of Dr. J. R. Haag, Department of Chemistry, Oregon State College, citric acid was tried for this purpose. Fortunately, this reagent makes the tissue again susceptible to differential staining with trypan blue. The procedure for formalin-fixed material is: (1) to prepare the peelings from the fixed material, (2) to place them directly into a 10% solution of citric acid for 1 to 6 hours, (3) to place them in the stain.

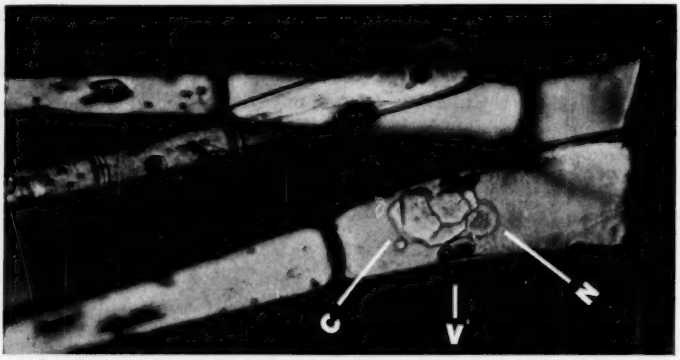

Fig. 1. Differentiation by Test 2 of tobacco mosaic virus evidence in *Lycopersicum trichome*. C, hexagonal crystal; V, compacted-alveolar viroplast; N, nucleus of the cell.

The concentrated dye may be used as directed below but 30 minutes in 0.05% usually gives an excellent preparation.

The concentrated 0.5% solution of trypan blue may be used to good advantage to stain material fixed in formalin when attempting Test 1. The average time required is 4 minutes. Moreover, living material known to contain virus evidence that reacts positively to trypan blue, may be quickly stained in the concentrated solution, the time being reduced to approximately 3 minutes.

Peelings from pubescent leaves make beautiful preparations by the method used in Test 2 if the cover is firmly flattened down and the tissue left in the trypan blue stain during examination. Since the

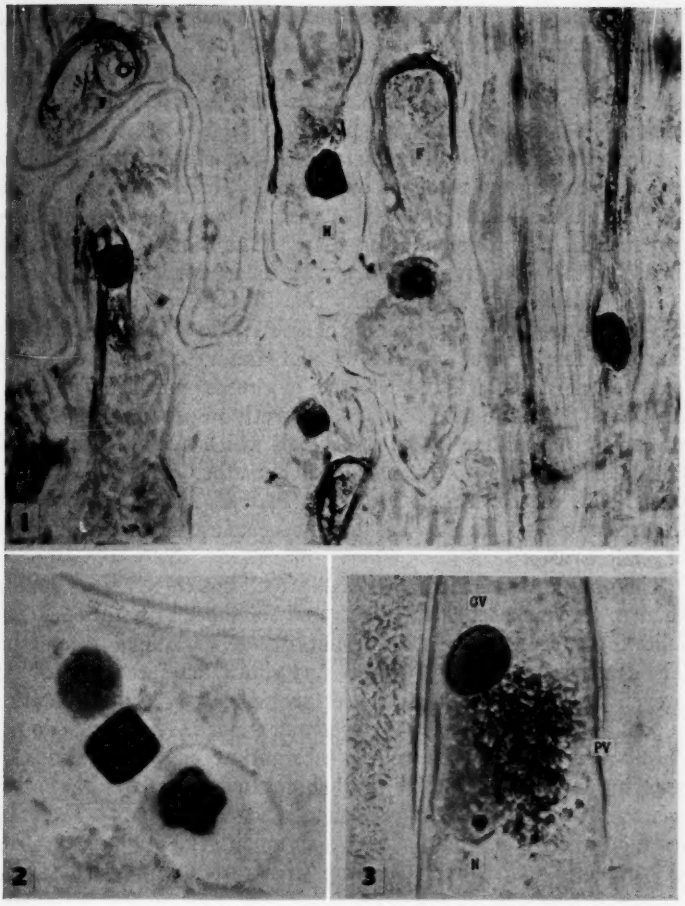

Plate I

1. Trypan blue demonstration of virus evidence after formalin fixation of peeling from lily leaf infected with latent virus complex. F, long curved fibrillae; V, fibrillate-granular viroplast; N, nucleus. ×350.

2. Demonstration of isometric crystals in Bean Virus 2 infection by Test 1 method applied to living leaf material. The proportionate stain absorption renders the large crystal darkest, the rosette of crystals in nucleus lighter, while the disintegrating plastid remains unstained. ×3000.

3. Test 1 applied to peeling from living tulip leaf infected with tulip virus 1. Note proportionate stain absorption. N, nucleus; CV, compacted viroplast; PV, particulate-reticule viroplast. ×400.

stain does not color the cell walls, the effect approximates a deep blue darkfield in which the trichomes stand out as brilliantly illuminated objects. (Fig. 1.) The progress of Test 2 can be followed in trypan blue mounts and the tissue removed when a desirable endpoint has been reached in the epidermal cells.

Permanent mounts can be made from formalin-fixed material by following the dioxan whole-mount technic (McWhorter and Weier, 1936). They should be deeply stained in an aqueous solution of trypan blue before dehydrating them. The dioxan process yields mounts with a refractive index favorable for examination of cell parts.

Illustrations and Suggestions. The illustrations accompanying this article show selective staining characteristic of these tests. The sharp staining of virus evidence without staining other cell contents, except nuclei, facilitates the detection of viruses by utilizing cytological clues. For example, Test 1 was directly responsible for the discovery by the author (1941) of crystals which average less than a micron in diameter in green chlorenchymatous cells of leaves infected with certain legume viruses. (Plate 1, fig. 2.) It is hoped that selective staining may become of specific use in the classification of plant viruses.

The procedures seem almost "fool proof" but there are, of course, chances for unexpected variations and for misinterpretation. The following suggestions relate to points where variations may occur. The detergent must be used for a very short time (seconds only) to prevent too rapid disintegration of protoplasts. No interpretation is possible until the nuclei have been well stained. Altho fats do not absorb trypan blue, it is well to substantiate one's conclusions by applying selective fat stains so that unusual elaioplasts cannot be mistaken for protoplasmic formations related to viruses. Formalin fixation involves coagulation that may modify the original structure of viroplasts. Continual checking with carefully prepared living mounts is always advisable.

(1) Bawden, F. C. 1939. Plant Viruses and Virus Diseases. 272 pp. Chron. Bot. Co., Leiden, Holland. (Contains an excellent discussion of the relation of inclusions to viruses.)

(2) McWhorter, F. P., and Weier, Elliot. 1936. Possible uses of dioxan in botanical microtechnic. Stain Techn., **11**, 107–17.

(3) McWhorter, F. P. 1940. Separation of tulip 1 virus from lily-latent by cytological methods. Phytopath., **30**, 788.

(4) McWhorter, F. P. 1941. Isometric crystals produced by *Pisum* virus 2 and *Phaseolus* virus 2. Phytopathology **31**, 760–1.

THE GERMINATION AND STAINING OF BASIDIA IN GYMNOSPORANGIUM

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ABSTRACT.—**A procedure is described for germinating and staining rust teliospores on the slide. The spores are germinated on slides in a damp chamber, about 3 hours being required for the production of sporidia. The material is killed by inverting slides over osmic acid fumes for a few minutes. Germinated spores are then allowed to dry on the slide, thus becoming fixed to the slide in a gelatin produced by the breaking down of their own stalks during germination. No other fixative is required. Material must be thoroly dehydrated in the alcohols (one or more hours in each of the higher alcohols); returned to water; mordanted for 2-3 hours in 4% iron alum; stained for 2-3 hours in 0.5% aqueous solution of Heidenhain's hematoxylin; destained in 2% iron alum. The material is passed back thru the alcohols and mixtures of xylol and absolute alcohol (1:2, 1:1, 2:1) to xylol and mounted in balsam. The method is particularly satisfactory for the *Gymnosporangium* rusts, which have telia very readily gelatinized. The details of germination are preserved intact, as in nature, and many details of nuclear division are excellent.**

In the study of germinating teliospores of *Gymnosporangium clavipes*, a rust with the telial phase on red cedar, a very convenient and successful method has been devised for germinating the teliospores and staining the nuclei in the division stages in the basidia and sporidia. By this method, which takes advantage of the natural gelatin in the teliospore stalks to fix the material to the slide, one of the greatest difficulties in handling germinating basidia in this group of rusts is overcome, i.e., the basidia are not torn away from the teliospores and the basidiospores are not broken off from the basidia. Teliospores of other rusts were germinated in drops of water on slides almost a hundred years ago, but (so far as the writer knows) this is the first time anyone has used the natural gelatin in the telial stalks to glue the germinating spores on the slide.

The teliospores are collected in early spring on cedar branches, where they occur as bright orange pustules on slightly swollen areas

[1]The writer is grateful to Professor J. N. Couch for helpful suggestions during this work.

of the branches. It is necessary that the rust be collected while still dry, and before the rains have caused a gelatinization of the telia. These spores will give good germination results for at least two weeks after being brought into the laboratory.

On a clean slide a single drop of distilled water is placed. With a needle a small mass of teliospores is teased out of a mature pustule and placed in the drop. The spores are then spread out with the needle so that they do not remain together in one mass, but lie in a more or less even layer. The slide is now transferred to a damp chamber for germination.

The water in which the teliospores are placed soon causes the spore stalks to gelatinize so that the spores come to lie in a gelatinous matrix on the slide. Spores germinate well at room temperature. After about three hours in the damp chamber they are usually ready to be removed. An examination under the microscope will show the presence of abundant basidia in the process of producing sporidia.

Before the procedure is continued, the germinating spores should be stirred thoroly with a needle in order to make the basidia lie on their sides. Otherwise the sterigmata with their sporidia will point straight upwards and will be unsatisfactory for study. The stirring process does not appear to injure the basidia in any way and does not dislodge the sporidia unless they are ready to be discharged. If the spores are still too closely massed together, they should be spread out into a thinner film, with more water added to facilitate this, if necessary. The natural gelatin produced by the spore stalks is the only substance required by this procedure for fixing the spores onto the slide.

The material is now ready to be killed. It is desirable to use a quick killing agent and one which does not require the slide to stand in liquid medium; otherwise the spores might be quickly dislodged from the slide. A killing chamber employing fumes of osmic acid is most effective. A watch glass is placed in a Petri dish and a few drops of 1–2% osmic acid are put into the watch glass. The slide, with the material on it still moist, is inverted over the osmic acid in the watch glass, and the dish is closed. The fumes of the acid kill the germinating spores quickly and the slide may be removed after 4–5 minutes in the killing chamber.

The slides are now placed out in the open and allowed to dry. The drying is accomplished usually within half an hour, the time varying with the amount of moisture used. The gelatin hardens and fixes the spores onto the slides. This does not harm the basidia, for the gelatinous film around them protects them from desiccation.

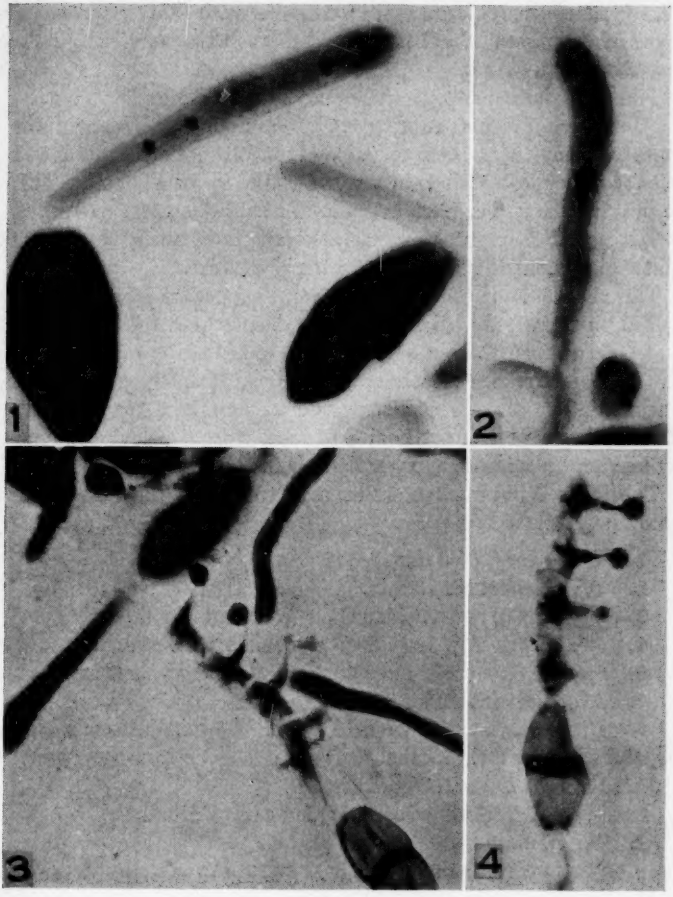

Plate 1. Photomicrographs of germinating teliospores of *Gymnosporangium clavipes.*

Fig. 1. Telophases of the second nuclear division in the basidium. ×1000.

Fig. 2. Anaphase of the first nuclear division in the basidium. Figure also shows a discharged binucleate sporidium. ×1000.

Fig. 3. Basidia, one of which is producing sporidia. ×500.

Fig. 4. Basidium producing sporidia, with nuclei passing into the sporidia. ×500.

This process occurs normally in nature between rainy periods. The material may even be left for several days in this condition without any obvious ill effects.

The procedure following the osmic acid treatment lays emphasis on thoro dehydration, subsequent to employing Heidenhain's iron alum hematoxylin staining technic. It will be noticed that slides are left for only short periods in water and the lower alcohols. This is to prevent the spores from becoming dislodged. After passing thru 50% alcohol, however, there is little danger of losing much of the material thruout the remainder of the procedure.

The necessity for thoro dehydration is apparent from results obtained by varying the length of time in the alcohols. When the slides were run up thru the alcohols at 10–15 minute intervals, there were left in the cytoplasm certain stainable inclusions which obscured all nuclear details after treatment with iron alum hematoxylin. What these inclusions are is not known to the author. They are removed by leaving the slides for longer periods in the higher alcohols.

The steps following the osmic acid treatment may be listed as follows:

1. Water for 2 or 3 min.
2. 10% alcohol for 15 min.
3. 20%, 30%, and 40% alcohols for 30 min. each.
4. 50%, 60%, 70%, and 80% alcohols for 1 hr. each.
5. 95% alcohol for at least 3 hr. Slides may be left overnight.
6. Absolute alcohol for 1 hr.
7. Return thru the alcohols to water at 15-min. intervals, bleaching the slides in 50% alcohol containing a little hydrogen peroxide (10 cc. H_2O_2 to 100 cc. 50% alcohol).
8. Water for 20–30 min., changing several times.
9. 4% iron alum for 2–3 hr.
10. Water for 15 min., changing several times.
11. Heidenhain's hematoxylin (0.5% aqueous solution) for 2–3 hr. A longer time is undesirable, as it makes destaining of the cytoplasm more difficult.
12. Water for 30 min., changing several times.
13. Dehydrate, passing thru the alcohols at 15-min. intervals.
14. Mixtures of absolute alcohol and xylol in ratios of 2:1, 1:1, 1:2 for 10 min. each.
15. Xylol for 30 min.
16. Mount in balsam.

The nuclei should now be stained so that they stand out distinctly in various stages of their history from early prophase in the basidium

to their passage into and division in the sporidia. They show up as black figures against a light cytoplasmic background. We have made a large number of slides showing distinct spindles during nuclear division in the basidia and in the sporidia. The technic is not one designed to show up individual chromosomes well enough to be certain of their form and number, but definite chromosomal units can often be seen on the spindles.

Figures 1 and 2 show the nuclei in the basidia in the process of meiotic division. The former illustrates the second meiotic division in telophase, and spindle fibers can be seen between the daughter nuclei. Figure 2 shows the anaphase of the first meiotic division. Figures 3 and 4 show basidia giving rise to sporidia. In figure 4 two nuclei were caught in the process of passing thru the sterigmata into the sporidia.

In several ways this technic is decidedly superior to the more complicated methods previously used in making basidial preparations of this group of rusts. By taking advantage of the natural gelatin produced by the stalks of the teliospores to fix the material to the slide it is possible to kill and stain material exactly in the stages desired. Furthermore, by avoiding a liquid killing agent followed by washing, the basidia remain attached to the teliospores and the sporidia remain attached to the basidia. Finally the injury to the protoplasmic structures is so slight that with appropriate staining excellent nuclear figures are obtained.

ERRATUM

Vol. 16, No. 4 (Oct. 1941) p. 152

A correction should be made in the article by Lindsay S. Olive, on The Germination and Staining of Gymnosporangium Basidia:

In the procedure for treatment and staining listed on this page, the following steps should be inserted between steps 11 and 12:

a) Water for 15 min., changing several times.

b) Destain in 2% iron alum until cytoplasm has cleared and nuclei are distinct.

PASTERNACK'S PARAFFIN METHOD MODIFIED FOR PLANT TISSUE[1]

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ABSTRACT.—**This method for preparing paraffin sections of plant material is a modification of Pasternack's one-hour method for animal tissues. Fixation in Randolph's CRAF fixative is hastened by heat and increased vapor pressure obtained by the use of screw top vials. Dehydration with Zirkle's butyl alcohol series likewise is hastened in the same manner. The rapid penetration of paraffin by the use of ½ paraffin and ½ butyl alcohol in heated screw top vials shortens the embedding process. Sections are held on the slide thru staining by albumen fixative and a coating of 0.2% celloidin in absolute alcohol and ether. Good penetration with freedom from shrinkage or distortion is obtained and root tip chromosome counts can be made in approximately 3 hours.**

Pasternack[2] has devised a one-hour technic for the preparation of paraffin sections of animal tissues. A combination of Pasternack's technic with existing conventional plant methods has yielded a schedule which in our work with the *Bromeliaceae* enables us to obtain root tip chromosome counts from paraffin sections in approximately 3 hours. This method combines good penetration with freedom from shrinkage or distortion of the tissues and the chromosomes are well separated.

The time-saving features of this schedule over the conventional methods used in plant cytology make it seem worth while to call it to the attention of other investigators.

Rapid penetration of the root tip tissues is obtained by heating the reagents as indicated in the schedule below:

1. *Fixing.* Heat root tips to 80° C. in a well stoppered or screw top vial containing Randolph's CRAF fixative (solution A: chromic acid 1 g., glacial acetic acid 7 cc., water 92 cc.; solution B: neutral formalin 30 cc., water 70 cc.) and keep at this temperature for 10

[1]Published with the approval of the Acting Director as Miscellaneous Paper No. 35 of the Pineapple Experiment Station, University of Hawaii.

[2]Pasternack, Joseph G. A reliable one-hour method for the preparation of paraffin sections of tissues. Amer. J. Clin. Path., Tech. Suppl., 4, 3–13. 1940.

minutes. Wash in several changes of warm water, 15 minutes minimum. Blot dry and wipe off any adhering soil particles.

2. *Dehydration.* Run the root tips thru Zirkle's[3] butyl alcohol series.[4] (A 5-minute treatment in each butyl alcohol step is sufficient with the material used in this experiment.)

A. Butyl I (10 cc. *N* butyl, 43.5 cc. 95% alcohol, 46.5 cc. water).
B. Butyl II (20 cc. *N* butyl, 54.0 cc. 95% alcohol, 26.0 cc. water).
C. Butyl III (35 cc. *N* butyl, 54.0 cc. 95% alcohol, 11.0 cc. water).
D. Butyl IV (55 cc. *N* butyl, 43.5 cc. 95% alcohol, 1.5 cc. water).
E. Butyl V (75 cc. *N* butyl, 25.0 cc. 95% alcohol).
F. Normal butyl I.
G. Normal butyl II.
H. Normal butyl and paraffin (1:1), 10 minutes.

3. *Embedding.* Place material in paraffin in a paraffin oven at 60° C. for a minimum of 15 minutes, preferably in a vacuum container; if convenient, leave in the oven during the lunch hour. Embed in paraffin and immediately place in ice water. Cut sections 5 to 10 μ.

4. *Mounting on slide.* Affix to slide with Meyer's fixative. Wipe excess water from back and edges of slide. Put on hot plate, 50° C. for 5 minutes. After melting, remove paraffin with xylene from a dropping bottle. Before the xylene evaporates, cover the section with 0.2% celloidin in absolute alcohol and ether in the proportion 1:1, using the ordinary dropping bottle. Drain off excess fluid and blow to hasten evaporation. Immerse the slide immediately in water to harden the celloidin. If done with care no undue shrinkage results from these steps. The extremely dilute concentration of celloidin leaves no trace on the finished slide.

5. *Staining.* Stain 3–10 minutes with a 1% aqueous gentian violet (Newton), followed by the iodine, alcohol, clove oil, xylol, balsam series.

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[3]Zirkle, Conway. The use of *N*-butyl alcohol in dehydrating woody tissue for paraffin embedding. Science, **71**, 103–4. 1930.

[4]Reagents are preheated to 56–60° C. Increased vapor pressure is obtained by screwing the lids on the vials as soon as the roots are inserted; the use of 6 to 8 cc. of reagent in 20 cc. vials is recommended.

AN APPARATUS FOR WASHING TISSUE

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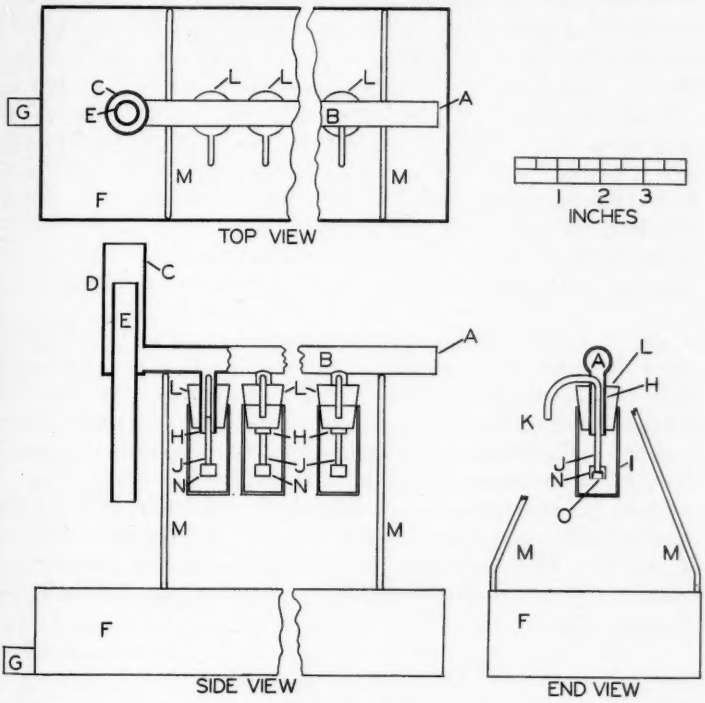
The apparatus shown below, which can be constructed in the ordinary departmental shop, has been found useful for washing large numbers of individual specimens preparatory to making histological



sections and is particularly applicable in handling successive portions of relatively large structures which are to be studied in series.

In operation, water from the laboratory faucet enters a horizontal brass tube B, at A, by means of a hose not shown in the figure. The faucet is adjusted so that water fills the tube B, and the vertical tube C where a considerable portion overflows at D thru tube E into the

pan F and out to the drain thru tube G and a hose not shown in the figure. This arrangement provides a constant head of water regardless of small variations in the tap water pressure. The specimens to be washed are placed in glass vials connected to the apparatus by the rubber stoppers L which normally remain attached to the tubes H. The way in which the water flows while washing the specimens can best be seen by examining the drawing at the lower right of Fig. 1, labeled "End View". Part of the water in tube A is diverted thru H into the glass vial I, then up thru the screen O, which is soldered to an enlarged opening N, which in turn is soldered to the eighth inch copper tube J. The water leaves this tube at K, falls into the tray F and finally leaves at G with the water from the other vials and that from E. The rods M attach the tube B to the pan F.

In the figure only three vials are shown altho as many as may be desired may be attached to the tube B. In the model used by the authors there were twenty vials. The entire apparatus is made of brass with the exception of the rubber stoppers L, the copper tube J and the glass vials I. All joints except those made by the rubber stoppers, are soldered. The drawing has been made to the scale shown at the top right and for the sake of clearness the vertical tubes C and E have been omitted from the end view.

The best way to assemble the apparatus is as follows: (1) drill the holes for tubes H, in tube B; (2) solder tubes H in place; (3) drill holes in tubes H for tubes J making them somewhat larger than the actual diameter of the latter; (4) the eighth inch copper tubes J are soft and easily bent so that, starting with straight tubes, one end is inserted in the appropriate hole in H, and with a combination of pushing and bending it is pushed inside H and finally bent with the fingers to the form shown in the end view; (5) the joint between J and H is then soldered. No particular order of assembly or instructions are needed for putting together the remainder of the apparatus.

PERMANENT STAINED PREPARATIONS OF THICK BLOOD FILMS

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ABSTRACT.—**Fixing thick films in alcoholic solution of dye after the usual staining-and-laking procedure preserves the appearance of parasites and blood elements very similar to that of the usual thick films (not fixed) for the diagnosis of malaria and relapsing fever.**

Procedure recommended: Films are stained and laked for 15 minutes in diluted Giemsa—1 to 3 drops of stock solution (0.4 g. in 60 ml. equal parts absolute methyl alcohol and glycerin) per ml. distilled water; rinsed in water and allowed to dry. They are then immersed in, or flooded with, May-Grünwald's stain (0.5% in absolute methyl alcohol) for 30 seconds, rinsed in water and allowed to dry. Solutions of MacNeal's tetrachrome stain in methyl alcohol and glycerin may be substituted for Giemsa and a solution in methyl alcohol may be substituted for May-Grünwald. With slight modification of the procedure, both thick and thin films on the same slide may be stained together.

Films stained and fixed as described, and mounted in Diaphane, have shown no evidence of fading in 3 years.

The thick blood film technic has been very useful in the diagnosis of certain infectious diseases, particularly malaria and relapsing fever. Unfortunately, films stained by the usual procedure (Barber and Komp, 1929), in which laking of the red cells and staining the remaining blood elements and parasites is accomplished by diluted Giemsa stain, are not permanent and one can count on fading within a few months depending on climate and other conditions. Covering films with liquid petrolatum or with petroleum jelly to prevent fading is often inconvenient.

In order to attain fixed preparations with staining properties similar to those of films stained in the usual manner, a number of laking and fixing agents were tried with dried thick films of blood containing the parasites of tertian malaria (*Plasmodium vivax*) or malignant malaria (*P. falciparum*). Staining with diluted Giemsa (or other blood stains) was unsatisfactory in one or more respects following treatment with: (1) 0.5 to 1% glacial acetic acid and 1% formalin (Ruge, 1903); (2) 2% glacial acetic acid and 0.4% tartaric acid—

also followed by methyl alcohol (Knowles, 1931); (3) acid alcohol—0.5 to 3% hydrochloric acid (James, 1911) or 5% acetic acid (Henson, 1913); (4) diluted ethyl alcohol, 10% to 70% (Vaillant, 1919); (5) acetone, water, alcohol (Bramachari and Sen, 1928). Similarly poor staining—whether by Giemsa or other technic—followed treatment of the films with simple laking agents such as distilled water, saponin in water or physiological saline, 5% glycerin and 5% methyl alcohol in water, ethyl acetate, etc. The characteristic staining of blood elements and parasites observed in films laked and stained in diluted Giemsa was altered unfavorably by *previous* treatment with any of the laking or fixing agents.

Fixation of films with alcoholic solution of dye *after* the usual staining (and laking) was found to yield satisfactory results, even tho the films were stained slightly different. A good many comparative trials were made with a number of blood stains in methyl alcohol and in equal parts of alcohol and glycerin. Solutions of Giemsa,[1] MacNeal's tetrachrome,[2] Wright,[3] azure-II-eosine,[4] May-Grünwald[5] and several non-commercial samples of dye were prepared in absolute methyl alcohol (0.15 g. dry stain per 60 ml., May-Grünwald 0.3 g. per 60 ml.) and in equal parts of absolute methyl alcohol and glycerin (0.4 g. dry stain per 60 ml. solvent) on several occasions and tested on blood films from various sources. Dried thick films were first stained-and-laked according to the usual technic—i.e., immersed in diluted alcohol-glycerin stain (1–3 drops of stock solution per ml. distilled water[6]) for fifteen minutes, very gently rinsed in distilled water and allowed to dry. Films stained by a given dye were then fixed with one or another of the alcoholic solutions of the several samples, either by immersing the slide or flooding it for a minimum period, rinsed in water and allowed to dry. Most satisfactory results were obtained with a period of fixation of about 30 seconds.

Most of the dyes stained the parasites at least well enough so that they could be recognized. Since Roe, Lillie and Wilcox (1940) have determined the particular dye components and their proportions which stain malarial parasites most satisfactorily, a detailed report of the relative merits of the samples used in this work is not needed. It may be stated, however, that the chief difficulty with

[1]Grübler's, NGe–1, NGe–2, NGe–3, NGe–5, CGe–1, LGe–1.

[2]NMn–9, three samples.

[3]NWr–12, CWr–11.

[4]Nat. Aniline & Chem. Co. No. 8356.

[5]Nat. Aniline & Chem. Co. No. 7598; Hartman-Leddon Co.

[6]Buffer is unnecessary for thick films if the salt content of the distilled water is properly low.

certain samples (especially Grübler's) was the darkly stained background of the film. And, contrary to expectation, two samples of May-Grünwald stained the parasites well in thick films, but poorly in fixed thin films.

Fixation with alcoholic solution of dye, after the usual staining, yields satisfactory preparations provided it does not destain the parasites or blood elements. Hence the limited period for fixation and the importance of saturated alcoholic solution of the stain used for this purpose. Irrespective of the type of stain used first, fixation with alcoholic solutions of May-Grünwald and of Tetrachrome usually gave somewhat better results than with alcoholic solutions of Giemsa, azure-II-eosine and Wright. To list all the details of results of the many combinations of stains and fixations with the samples of dyes used in this study would probably defeat the purpose of presenting a simple, reliable method. However, on the basis of these comparative tests a few definite procedures can be recommended: (1) staining-and-laking with Giemsa (which is most commonly used for thick films), followed by fixation with May-Grünwald (0.5% in methyl alcohol); (2) staining-and-laking in an alcohol-glycerin solution of tetrachrome (which has yielded results as good as Giemsa), followed by fixation with alcoholic solution of the same dye; (3) slight modification of the procedure for use with slides which have both thick and thin films. Description of the procedures follows.

1. Thick films, prepared by spreading one large drop of blood on a slide over an area of about 1 cm. diameter and allowing it to dry, are immersed in diluted Giemsa stain—1 to 3 drops of stock solution (0.4 g. in 60 ml. equal parts absolute methyl alcohol and glycerin) per ml. distilled water for 15 minutes.[7] The film is then gently dipped into distilled water momentarily and allowed to dry. Fixation is accomplished by immersing the film or flooding it with May-Grünwald (0.5% in absolute methyl alcohol) for 30 seconds. The film is rinsed in distilled water and allowed to dry.

2. The dried thick film is immersed in diluted alcohol-glycerin solution of tetrachrome—1 to 3 drops of stock solution (0.4 g. in 60 ml. equal parts absolute methyl alcohol and glycerin)—for 15 minutes, dipped in distilled water and allowed to dry. The film is fixed for 30 seconds with alcoholic solution of the same dye (0.15 g. in 60 ml. absolute methyl alcohol), rinsed in distilled water and allowed to dry.

3. Thick and thin films of the same blood are frequently spread on

[7]Altho many authors specify 30 minutes or more, the writer has found that films may be satisfactorily stained and laked within as short a period as 5 to 10 minutes.

the same slide in diagnostic or survey work. The films on such a slide can be stained separately if a line of paraffin is painted between the films. A more convenient method is to stain the two films on the one slide as follows: slides are placed vertically in just enough diluted Giemsa stain to cover the thick films without coming into contact with the thin films, allowed to remain for 15 minutes, dipped momentarily in distilled water (without allowing the water to come in contact with the thin films), and allowed to dry. The slides are placed (individually) in a vial containing sufficient alcoholic solution of May-Grünwald to cover both films for 30 seconds and rinsed very quickly (incompletely) in water and then placed in diluted Giemsa in a Coplin jar for 15 minutes. The preparations are washed briefly by running distilled water into the jar (to avoid precipitate on the films) and allowed to dry. Alcohol-glycerin solution of tetrachrome may be substituted for Giemsa, and alcoholic solution of tetrachrome for May-Grünwald. The last treatment of the thick film with diluted Giemsa apparently does not alter the result in any respect.

By observing the ordinary precautions of preparing blood films as, e.g., the use of clean, grease-free slides of good grade (minimum free alkali), and by using satisfactory grades of stains (certified, if possible) and solvents, one can obtain very good preparations which are "permanent". Mounting stained blood films in Diaphane[8] reduces the tendency to fade due to condensation on the slides which is especially troublesome in a humid climate. (Caution: temperatures above 40° C. are likely to cause fading. Balsam invariably causes stained blood smears to fade.) The writer has thick-and-thin films stained by the third method described above, mounted in Diaphane, which have not shown any tendency to fade in 3 years.

In appearance the thick films prepared by any of the three procedures are quite similar and differ from the usual stained thick film, which is not fixed, only in minor respects. In satisfactory preparations, the red (chromatin) and blue (cytoplasm) stains of malarial parasites are retained undiminished and well defined, and the blood elements appear about the same. The chief difference from the usual "unfixed" thick film is the lighter, somewhat violet background in the fixed films. Apparently the short process of fixing removes the basophilia, or polychromatophilia, a fact which is often very noticeable in thick films stained in the usual manner. In fact, Barber and Olinger (1931) were able to estimate the grade of anemia by the density of the "blue clouds" in such films.

The same procedures were tested with rat's blood containing the

[8]Will Corp., Rochester, N. Y.

spirochaetes of relapsing fever (*Borrelia novyi*)[9] and with dog's blood containing the microfilaria of dog heart worm (*Dirofilaria immitis*). The spirochaetes appeared the same as in ordinary thick films, but were rather more easily recognized because of the somewhat clearer background. In dog's blood the microfilariae took a more intense and differential stain and, again, the background was lighter than in films not fixed.

The special value of permanent preparations of stained thick blood films would seem to be (1) for certain research purposes as, e.g., counting parasites, and (2) for use in instruction relative to the importance of thick films in the diagnosis of certain diseases and the appearance of parasites in such preparations. For experienced observers, the thick blood film offers the most sensitive, the most certain, and the simplest means for the diagnosis of malaria and relapsing fever. The fact that it is barely appreciated by the many individuals who are not specialists but frequently have occasion to use it, is undoubtedly due, in part, to the lack or paucity of suitable material for instruction. For this reason the author sought a method which would preserve, insofar as possible, the appearance of the thick film stained in the usual manner for diagnostic purposes, and hopes that the procedures described here will contribute towards a more general acquaintance of students, practitioners and investigators with the appearance of parasites in such preparations. As the technic is simple and has been fairly well standardized by Barber and Komp (1929) and by Roe, Lillie and Wilcox (1940), while the stains used for this purpose are certified by the Commission on Standardization of Biological Stains there is every reason to look forward to a greater appreciation and wider use of the thick film.

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[9]The writer is indebted to Dr. Vernon Schuhardt of the Department of Bacteriology at the University of Texas, Austin, Texas, for supplying this material.

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PREPARING PERMANENT DEEP CHAMBER MOUNTS OF VARIABLE DIMENSIONS

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ABSTRACT.—**A rapid and simple method for the preparation of permanent deep chamber mounts of variable size, shape and depth is described. The chamber sides are made with aluminum wire bent on a form of the proper size and shape. Aluminum wire is nicely adapted for use due to its pliability and clean appearance. It undergoes no apparent change in contact with the mounting medium. Depth of the chambers is determined from the gauge of the wire. Clarite, used as the mounting medium, is prepared as a thick solution in toluol (70-75% of clarite by weight). The resulting preparations are crystal clear, colorless, and according to the work of other investigators, do not become acid with age.**

The need for a simple and rapid method of preparing a large number of permanent deep chamber mounts of cleared thick sections of tissue prompted the development of the technic described below.

Numerous methods have been used for making mounting chambers for such things as small whole specimens and thick sections. Rings punched from various plastics, thick hollow chamber slides, covers supported by small broken bits of glass, slides with moats built up with rings of varnish or cements, have all been advocated and used for a long time. Some of the methods such as the hollow-chamber slides and commercially made mounting rings are quite expensive. Other technics produce excellent results but are tedious and slow.

The mounting medium used to fill such chambers has nearly always been Canada balsam or gum damar. It is common knowledge that Canada balsam, a deep yellow to begin with, darkens further on aging. Gum damar, while somewhat paler, also darkens. Either of these resins in a deep chamber produces an undesirable color. While it is true that this color makes no appreciable difference in the visibility of the preparation (this being mainly a function of the refractive index of the mounting medium compared to that of the tissue), it is obvious that a colorless mounting medium is technically more desirable, provided it meets other necessary requirements.

In the past four or five years a number of synthetic materials have been suggested for use as mounting media. Skiles and Georgi (1937) discussed the use of two synthetic resins as mounting media. These

resins, "Vinylite" and "Pontalite" (Lucite), were used for bacterial slide mounts and permanent mounts of moulds. They believed that these resins were superior to Canada balsam. Richards and Smith (1938), however, found Lucite unsatisfactory when used under a cover glass. Certain stains showed considerable fading and air bubbles appeared as the preparations dried. Kirkpatrick and Lendrum (1939) reported successful results with distrene 80, a synthetic resin to which they added tricresylphosphate to act as a plasticizer in reducing shrinkage from drying. This material was stated to be water-clear in xylol solution, of a constant pH, and was claimed not to cause fading of delicate stains. Isobutyl methacrylate, recently suggested as another synthetic material suitable for use as a mounting medium (O'Brien and Hance, 1940), has had certain objections raised against it by Hamilton (1940) and Groat (1940) who stated that it caused fading of stains, distortion with drying and that the refractive index (1.477) was too low for most purposes.

Groat (1939) gave the first account of the use of clarite and clarite X as mounting media. He found them superior to Canada balsam and gum damar in all respects. His paper should be referred to for a detailed description of the properties of these substances.

Little mention has been made of the practicability of using such synthetic resins for permanent deep cell mounts. Mohr and Wehrle (1940) briefly mentioned the superiority of clarite over Canada balsam for whole mounts of flukes. Smith (1940) stated that in making whole mounts of thick objects clarite had to be used in dilute solutions (60%) and showed the serious drawback of needing frequent "fill-ins" while such preparations were seasoning. Thick solutions of clarite in toluol have been found entirely satisfactory for use in the deep chambers described in this paper.

In the past practically all deep mounts were prepared in a round chamber utilizing a circular cover slip. Such covers are very much more expensive than square ones. This fact becomes a disadvantage if many specimens are to be mounted. The depth of chambers has, in most cases, apparently been a matter of guesswork. However, the possibility of using a high power objective in the examination of thick sections depends on having no excessive depth to the chamber. The technic to be described allows the use of square, rectangular or round covers of any desired size, together with the precise regulation of the depth of the chamber.

Materials and Method

The deep chamber preparations are made by placing a ring or square of aluminum wire of the desired thickness on the slide, placing

the object to be mounted within its confines, pouring in the proper amount of thick clarite and carefully applying a cover glass. Cementing the wire to the slide is unnecessary as a thick solution of clarite does this neatly and with little leakage, provided the wire is flat on the slide.

Aluminum wire may be obtained in the following gauges:

Gauge (B. & S.)	Diameter in millimeters (nearest 1/100 mm.)
14	1.63
16	1.29
18	1.02
20	.81
22	.64
24	.51

From this table the depth of a chamber can be determined by using wire of proper gauge. Wire rings are most easily made. Square wire frames are a little more difficult to shape perfectly but with practice this can be done.

Wire rings. A metal or hardwood cylinder of the proper size, i.e., smaller than the cover slip diameter by two times the thickness of the wire, is clamped in a vise and a number of turns of wire wound closely and tightly around it near one end. This coil is carefully slid off and with cartilage shears (or some similar cutting instrument) a cut is made along one side of the coil, crossing all the turns of wire. This produces a number of exactly similar rings. These are then shaped individually in a small, smooth-faced vise so that they will lie flat on a slide.

Wire squares. The procedure for making square wire frames is identical to that for rings, except that a square metal shaft is used. For a perfect fit the size of the shaft is again the width of the cover slip minus two times the diameter of the wire.

When the wire is centered on the slide the chamber is ready for use. The tissue is transferred from toluol into the chamber and a thick syrupy solution of clarite in toluol (70% or a little more) is poured in to fill the chamber completely. Any trapped air under the tissue is teased out and the cover slip applied. The objection raised by Smith (1940) that rapid crust formation when using toluol solutions of clarite interferes with the proper application of cover slips to ordinary slides has found an answer in the deep chambers. Crusting over does occur in a few seconds time at room temperature. If the cover slip is lowered slowly, however, starting at one edge of the

chamber, the crust redissolves rapidly as the glass touches it and no air bubbles will be trapped. Apparently the exclusion of air allows toluol from below the surface to dissolve the crust.

Drying temperatures of 38° C. over a period of two months and 56° C. for a period of three weeks have caused no cracking or retraction of the clarite in the chambers. Exposure to sunlight daily for seven days has not damaged the mounts. Exposure to heat in a carbon arc microprojector for periods of twenty minutes did not alter the appearance of the clarite in the chambers.

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ACETIC-ORCEIN: A NEW STAIN-FIXATIVE FOR CHROMOSOMES

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Abstract.—**A new stain-fixative method for chromosomes, namely acetic-orcein, is described, which gives results that are equally good in fresh and permanent preparations. A 45% acetic and 1% orcein content is recommended as a standard solution. For salivary glands of *Drosophila* a 2% stain gives the best results, and with the two species *D. melanogaster* and *D. miranda* the acetic strength has been raised to 70% with advantage. The addition of chloroform proves necessary for hardening in species of *Sciara*. Acetic-orcein is equally good for rapid chromosome counts. For root tips the addition of 1 cc. of *N* HCl solution to 10 cc. of the standard solution together with gentle heating of the tissues in a drop of the mixture assists in the softening and separation of cells necessary for chromosome study. Orcein can also be used successfully in other combinations such as acetic-propionic or acetic-lactic. The latter is useful for making preparations that do not require ringing. Preparations so made keep from 7 to 14 days.**

Value of Orcein

The aceto-carmine method now so generally used for chromosome study has two shortcomings. It is not particularly selective, especially for small chromosomes, and preparations often deteriorate when made permanent.

The writer has recently discovered a more selective stain, namely orcein, another natural dye which can be used with acetic acid.[1] There is no reference in the literature to its having been used as a stain for chromosomes.

Acetic orcein is equally effective with fresh and permanent preparations. The staining is more easily regulated than with carmine and in very few finished preparations is any color retained by the cytoplasm. Coloration of the cytoplasm is a defect often noticeable in aceto-carmine preparations.

[1]Apparently it is difficult to obtain standardized samples of orcein free from all impurities. The two samples of dye used during the course of these observations were supplied by British Drug Houses. No difference in results was noted.

Acetic orcein has been used successfully with a number of plant and animal cells, and its selectivity can be seen in preparation of salivary gland chromosomes, where a band and heterochromatin structure is revealed more clearly than with other methods.

The keeping qualities of acetic orcein are good, altho periodic filtering is necessary with the solutions of higher dye-content, which tend to form a scum on the surface. This scum may be an oxidation product due to impurities in the dye.

Staining is good after acetic alcohol fixation, but becomes increasingly more difficult after storage in 70% alcohol. Tissues that have been pre-fixed should therefore be rinsed in 45% acetic acid previous to staining, and material stored in 70% alcohol should be left in acetic acid for one hour or more. No advantage has been found in the use of iron as a mordant.

To judge from preparations three months old, the stain has good keeping qualities. To obtain permanent preparations, however, one must remember that absolute alcohol is a solvent of the dye and one should therefore avoid mounting media (such as Euparal) which contain alcohol.

Technic

The schedule advised, which is similar to that used with aceto-carmine, is as follows:

A. Preparation of acetic orcein solution

The standard solution is 1% orcein in 45% acetic acid. It should be made as follows: Dissolve 1 g. of orcein in 45 cc. of hot glacial acetic acid (near boiling); leave till cold and then add 55 cc. of distilled water. Shake well and filter.

Modified solutions contain up to 70% acetic acid and 0.5-2% orcein.

B. Preparation of temporary slides (anther or testis)

1. Tease out cells in a drop of acetic orcein (0.5% orcein is often sufficient for large chromosomes). To secure even penetration, leave 30–60 seconds before placing the cover slip in position. Air bubbles should be avoided.

Note: The following special precaution is worth taking against loss of cells: Smear the cover slip thinly with Mayer's albumen, wipe off excess and then heat carefully over a low flame till a gray smoke is given off. Use when cool. Most of the cells will then remain attached to the cover slip.

2. Pass the slide carefully two or three times over a low flame; do

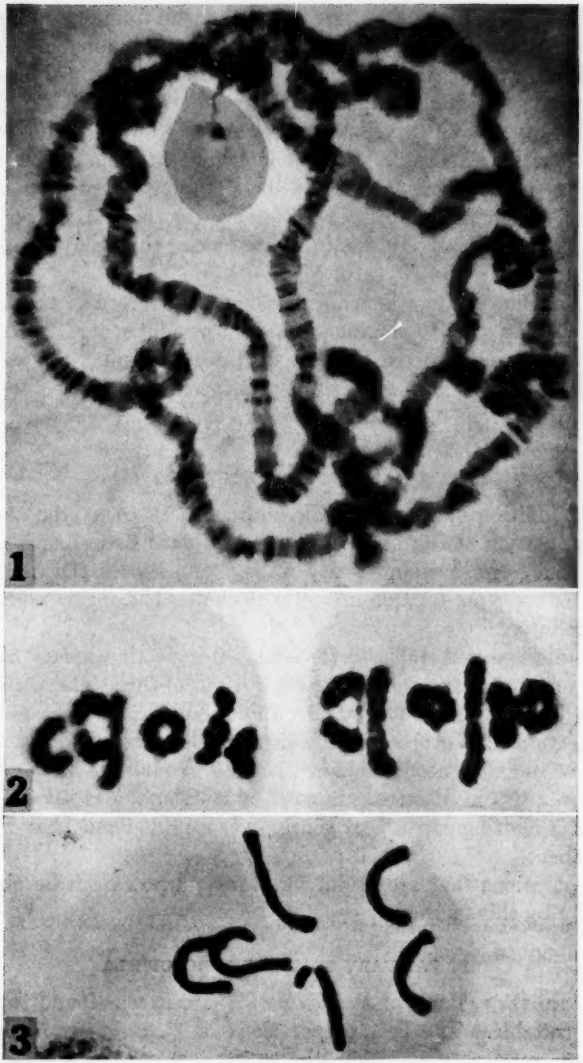

Fig. 1. *Drosophila* sp., salivary gland, showing nucleolus and organiser. Standard acetic-orcein solution, followed by acetic-orcein gelatin medium. ×900.

Fig. 2. *Tradescantia bracteata*, pollen mother cells. Half strength orcein. ×1,400.

Fig. 3. *Crocus balansae*, root tip, metaphase. Standard acetic-orcein solution plus *N* HCl. ×1,900.

not allow to boil. The heating of slides made from stored material needs extra care, for an excess of heat results in complete destaining.

3. If very flat plates are required they are best obtained by applying an even pressure on the cover slip under several thicknesses of blotting paper. The cover slip should not be allowed to move during this operation, and it is best done before heating. With aceto-carmine (perhaps on account of its temporary hardening action) Belling[2] recommended pressure after two or three days, but since tissues remain relatively soft in acetic orcein, this delay is unnecessary.

4. The slide can now be made permanent, or ringed as a temporary mount with a view to making permanent at a later date. It should, however, be remembered that, as with aceto-carmine, the intensity of the stain in temporary mounts will increase with age. For this reason they are best kept in a cool dark place.

C. MACERATED ROOT TIPS

The addition of 1 cc. *N* HCl solution to 10 cc. of the standard orcein solution assists in the softening of plant tissues such as root tips, young embryos and ovary walls. The tissues can be stained fresh, or after pre-fixation in acetic alcohol. The suggested method is as follows:

Rinse tissues in 45% acetic acid, after acetic alcohol fixation. Place in watch glass, containing a few drops of the solution, and heat gently 3–4 times over a low flame; do not boil. Slice the tissues into thin sections in a drop of fresh stain on a slide; crush with a scalpel or blunt end of a needle holder. Leave 3–4 minutes before placing the cover slip in position. Squash by blotting under several thicknesses of blotting paper, heat gently over a spirit flame, seal or make permanent.

Preparations that are useful for many purposes can be obtained quickly by this method.

D. SALIVARY GLANDS OF DROSOPHILA

For salivary glands of *Drosophila* 2% orcein was found to be the most suitable. The best preparations of *D. melanogaster* and *D. miranda* were obtained when the acetic acid content was raised to 70%. The following method is advised:

1. Dissect glands in Ringer solution or drop of acetic orcein. Leave in stain 5 to 10 minutes.

[2]Belling, J. 1930. The Use of the Microscope. McGraw-Hill, New York. See p. 242.

2. Dry stain accumulates by evaporation round the rim of the drop; remove this by carefully wiping with moistened blotting paper before placing the slip in position.

3. For flattening plates acetic orcein, on account of its softening effect, requires less pressure than acetic carmine.

E. SALIVARY GLANDS OF SCIARA

The following method was found to be most suitable:

Dissect out glands in 45% acetic acid and transfer to a drop of the standard acetic orcein solution containing 10% chloroform. Staining for 2–3 minutes is generally sufficient. Altho only a trace of the chloroform diffuses into the acetic acid, the bulk remaining as a layer at the bottom of the vial, this trace has a slight hardening effect on the tissues which enables one to obtain excellent preparations with no color in the background.

F. PERMANENT PREPARATIONS

1. Separate the slide from the cover slip by turning it face down in a ridged dish containing 10% acetic acid; after 3–10 minutes the cover slip will fall off.

2. Pass the slide and cover slip (with salivary glands all the material will remain attached to the cover slip if treated as above with albumen) thru the following solutions: 80% alcohol, 2 minutes; absolute alcohol, 2 minutes; two changes of cedarwood oil, 5 minutes in each (a longer time does no harm).

3. Recombine slide and cover slip by mounting in thick immersion oil, or balsam. Blot carefully to remove excess cedarwood oil, dry on hot plate before cleaning.

Uses of Orcein

1. Orcein can be substituted for carmine in Zirkle's gelatin combination[3] (none of Zirkle's other mixtures have been experimented with as yet). This orcein-gelatin combination was found to be most useful in showing the structure of the nucleolus and its attachment to the chromocenter in species of *Drosophila*. For this purpose it is recommended to be used after fixing and staining with standard acetic orcein solution, either before placing the cover slip in position, or after separation in 10% acetic acid, depending on the degree of

[3]Zirkle, C. 1940. Combined fixing, staining and mounting media. Stain Techn., 15, 139–53.

staining required. It can also be used for pollen mother-cells and other tissues. The modified formula is as follows:

Gelatin	10 g.
Sucrose	10 g.
Glacial acetic acid	50 cc.
Distilled water	60 cc.
Orcein	1 g.

The omission of stains from Zirkle's medium makes it an ideal ringing medium, which dissolves in the 10% acetic acid solution used for separation, should it be wished to make the slides permanent at a later date.

2. Orcein can also be used successfully in mixtures of propionic and acetic acids, or a lactic-acetic combination, consisting of equal parts of glacial acetic acid, lactic acid and distilled water plus 0.5% orcein. Preparations made with this mixture will keep without ringing from 7 to 14 days. The stain is a little slower in acting, but is intense enough after a few hours. The use of lactic acid was suggested by my former colleague, Dr. A. C. Fabergé.

IDENTIFICATION OF SPERMATOZOA IN CRIMINOLOGICAL INVESTIGATIONS

J. D. LAUDERMILK, *Pomona College, Claremont, Cal.*

In certain types of medico-legal examination, it is frequently of great importance to be able to make specific identification of spermatozoa. In the great majority of these cases, it is required that the cells should be isolated from dried material on cloth. Several procedures have been proposed and put into practice to this end. In general, these methods consume considerable time and are relatively complicated. The evidence requires much handling, and, as one of the leading authorities[1] says ". . . .From a long experience of this work, we are able to state that it is not so easy a matter as the student may imagine from the ordinary accounts in books. . . ." While relatively simple in theory, it becomes much more complicated in actual practice and sometimes operates to the chagrin of even the professional microscopist who is inexperienced in this particular work.

During the past several years, I have made many examinations of this type for the police and have been constantly aware of the drawbacks mentioned by the above authority. In view of this deficiency the following technic has been developed, which has invariably given satisfactory results. The schedule is applicable to stains at least a year old. In most cases this is a much greater lapse of time than will be required. The schedule follows:

1. Locate the stained areas under ultra-violet light. This is very easily done by fitting a *Corning* filter No. 587[2] to an ordinary three cell flashlight by means of a simply constructed housing. This filter transmits *soft* ultra-violet of from 3200 to 3900 Å. Under this illumination, stains resulting from seminal fluid, when dry, show up as sharp, bluish-white, clearly mapped-out areas which are highly fluorescent.

2. Having located the suspected areas, remove pieces of the cloth about 4 mm. square by cutting out with dissecting scissors. Where the suspected area is small, only half the sample should be taken, so that untouched material may remain for further examination if required. Prepare as many slides as there are samples taken.

[1]Glaister, John. 1938. Glaister's Medical Jurisprudence and Toxicology. 6th ed. E. & S. Livingstone (16–17 Teviot Place), Edinburgh. See p. 408, 2nd paragraph.

[2]Corning Glass Co. Corning, N. Y. *Heat resisting, red-purple ultra* No. 587.

3. In the center of each slide place a small drop of cold water. Shove the cloth square into the drop. If the cloth is simply placed on top of the drop, this is apt to spread and make a slovenly looking smear. Let the cloth soak for one minute.

4. Remove excess water with a piece of filter paper so that no overflow covers the square. The cloth should be wet but there should be as little excess water as possible. Add one drop of *boiling* distilled water and allow the preparation to stand for one minute. Remove excess water and repeat the operation three times.

5. After the third treatment, shred the wet cloth square with the needles until well teased out. The yarns should be well separated.

6. Allow the slide with the teased out yarns to dry on a hot-water plate. This can be extemporized from a plate of galvanized iron placed over an ordinary copper hot-water bath. When the slide is perfectly dry, remove the bulk of the yarns and fibers with needle and forceps. This operation generally requires about five minutes; a smear will remain.

7. Stain the smear for two minutes with a solution of basic fuchsin[3] containing 25 mg. of the dye in 10 cc. of water. Apply the stain with a dropper and spread out over the smear with a needle.

8. Wash the slide six times by flooding with distilled water squirted on above the stained smear by means of the dropper while the slide is held in an inclined position over the waste-jar. Dry again on the hot-water plate, mount in balsam and examine. Spermatozoa are deeply stained and show up sharply by this treatment.

After having had experience with practically all the generally used methods for isolation and identification of spermatozoa in cloth, I have found it to be practically useless to examine unstained material or temporary mounts. Where this type of evidence has to be exhibited in court, permanent mounts are absolutely necessary. By the schedule given above, and which may be completed in twenty minutes, I find that the cells are not only hardened so that the tails are preserved in many cases, but that the whole cells are fixed to the slide by the action of the boiling water on the albuminous material extracted from the stained cloth. Handling of the evidence is reduced to a minimum. Results have invariably proved satisfactory with stains on cotton and rayon more than a year old. There is only slight probability that any evidence will be lost in manipulation.

[3]Basic fuchsin, total dye content 82%. National Aniline & Chemical Co. Inc., New York.

NOTES ON TECHNIC

A Plasticized Polystyrene Mounting Medium. Slides mounted in synthetic resin media frequently retract badly under the coverslip in drying, an objection previously noted by Mohr and Wehrle.[1] Kirkpatrick and Lendrum[2] minimized this difficulty in their trials of synthetic mounting media by incorporating the plasticizer tricresyl phosphate, and found that a British-made polystyrene resin was satisfactory when so plasticized. An American-made polystyrene resin (sample No. XMS 10023) obtained thru the kindness of the Bakelite Corporation appears to be equivalent to that described by the British workers. Mounts set very hard with little retraction when dried at room temperature or in the paraffin oven if a slight excess of the mounting medium is left around the margin of the cover slip. Brain tissue stained with thionin for demonstration of Nissl substance has shown slight fading after one year. Routine pathological tissues stained with hematoxylin and eosin, and with Lillie's modified Masson, in Dr. M. M. Mason's laboratory, have not faded after five months. The older and dryer mounts remain water-white and become unusually clear, probably because of the higher refractive index (1.60–1.62) as compared with damar (1.52).

Preparation: suspend 60 g. of resin in a mixture of 100 ml. of toluene and 18 ml. of tricresyl phosphate, and place the stoppered container overnight in the paraffin oven. By morning the resin will usually have dispersed to form a water-clear syrup which is then decanted for use.

The resin is obtained as clean, dry, white pellets. The price is low: in 1940 approximately $0.68 per pound if purchased in small lots. The tri-*o*-cresyl phosphate was a gift of the Monsanto Chemical Company. A purified Eastman grade was also satisfactory; no experiments were conducted with the much less expensive technical grade. It should be noted that tricresyl phosphate is a violent poison if ingested.—S. H. Hutner,[3] Middlesex Univ., Waltham, Mass.

An Improved Dilution Fluid for Erythrocyte Counts. The following fluid appears more convenient than Hayem's or Toisson's

[1]Mohr, J. L., and Wehrle, Wm. 1940. Resins for sealing glycerin mounts. Stain Techn., 15, 174–5.

[2]Kirkpatrick, J., and Lendrum, A. C. 1939. A mounting medium for microscopical preparations giving good preservation of colour. J. Path. & Bact., 49, 592–4.

[3]Present address: 342 Commonwealth Ave., Boston, Mass.

for red cell counts: iodine, 0.3%; potassium iodide, 0.4%; sodium citrate ($Na_3C_6H_5O_7.2H_2O$), 1%. It is prepared as follows: dissolve the potassium iodide in 50 ml. of distilled water; add the iodine crystals, using gentle heat to accelerate their solution; then add the citrate, make up to 100 ml. and filter. With this fluid the erythrocytes are stained brown against a clear background, while leucocytes are destroyed. The iodine stain is easily removed from the walls of the counting chamber with the usual glassware-drying solvents. The erythrocytes are not altered in morphology or clumped even at the end of an hour's standing in the counting chamber. Exposure of the solution to direct sunlight over a period of ten days did not result in appreciable change.—Luis A. Vallarino, Department of Bacteriology and Immunology, Middlesex Univ., Waltham, Mass.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

COLE, ELBERT C. **Textbook of Comparative Histology.** 6 x 9 in. 396 pp. Cloth. 297 illustrations. The Blakiston Company, Philadelphia, Pa. 1941. $4.00.

Altho not so implied in the title, this book actually deals with animal histology only. Most of the book is a discussion, profusely illustrated with fine photomicrographs, of the histology of animal tissue and of the tissue-complexes making up the various systems of organs. The last three chapters, however, (65 pp.) deal with instruments and methods, thus bringing the book within the scope of these reviews. These three chapters deal respectively with: "The Use and Care of the Microscope"; "Methods of Recording Data"; "Histological Technique". The last of these chapters treats a large subject in a very brief manner; it appears more useful for reference purposes than as a guide to be followed by a laboratory worker.

MICROSCOPE AND OTHER APPARATUS

HERTZMAN, ALRICK B. **A simple, inexpensive, vertical illuminator for the student microscope.** *J. Lab. & Clin. Med.*, 26, 868. 1941.

There is need for vertical illumination for examining human skin capillaries and minute vessels in the frog. The following method is simple and efficient: Mount a pencil-flash-light bulb, Mazda 222, (operating on 2.2 v. current) in a screw type dial socket carried by a copper wire 10 in. in length, and ⅛ to ¼ in. in diameter. Clamp this to a ring stand. As a source of current, use dry cells or a filament transformer operating at 2.5 v. which may be reduced to 2.2 v. by a resistance wire in the lead to the bulb. The 2.5 v. current may be used to provide a brighter light for photomicrography.—*John T. Myers.*

JOHN, K. **Über ein neuartiges Integriergerät und seine Anwendung insobesondere in der Biologie.** *Zts. wiss. Mikr.*, 57, 163–8. 1940.

R. Fuess of Berlin has just put on the market an integrating device permitting direct differential area determinations under the microscope. It consists of a special mechanical stage coupled by a flexible shaft to a very compact, motor-driven, counting apparatus. The latter is equipped with seven push buttons, each placed adjacent to a cyclometer dial. A special grating is provided for the eye-piece. The instrument is operated by moving the mechanical stage in a horizontal direction following the guide lines in the eye-piece. When desired to estimate the area of various cell types, a push-button is arbitrarily assigned to each type and depressed while moving the object along the guide line. Vacant spaces and artifacts to be discounted are estimated on a special push-button, making it possible to estimate the comparative areas of various cell types in a given field. Volumetric measurements may readily be made by multiplying the areas obtained by the thickness of the section.—*J. M. Thuringer.*

KAUSCHE, G. A. **Ergebnisse und Probleme der experimentellen Virusforschung bei Pflanzen (mit übermikroskopischen Aufnahmen).** *Ber deut. botan. Ges.*, 58, 200–22. 1940.

The writer discusses the value of the electron microscope in the study of plant viruses. He points out that at a magnification of 16,000 diameters the so-called Stanley crystals appear to consist of bundles of smaller units arranged parallel to

the long axis. These smaller units have dimensions within the range demanded of virus molecules. The reaction of the virus with colloidal gold is shown in photographs. At the neutral point no reaction occurred. The addition of NaCl caused flocculation, while a gold sol acidified with HCl showed the gold particles laid down singly in the virus molecule or its aggregate. When an alkali is added, dispersion takes place due to the disintegration of the virus.—*Merritt N. Pope.*

SCHNEIERSON, S. STANLEY. **A simple, clean, cedar oil bottle.** *J. Lab. & Clin. Med.*, **26**, 1347. 1941.

The following bottle for immersion oil is clean and convenient: Take the dropper from an ordinary staining bottle with ground glass joint, and draw out the tip to a fine point, using the pilot light of a Bunsen burner. Cut off the tip leaving a very small opening of such a size that oil will not drop out by gravity. Fill the hollow part of the dropper with a Pasteur pipette and apply the rubber bulb. A controlled amount of oil can be dropped on a slide. No smearing, evaporation, or hardening of the oil will occur.—*John T. Myers.*

MICROTECHNIC IN GENERAL

GROAT, R. A. **Two new mounting media superior to Canada balsam and gum damar.** *Anat. Record*, **74**, 1–6. 1939.

Two new synthetic resins, Nevillite V and Nevillite No. 1, cycloparaffines or naphthene, are found to be superior to the natural resins Singapore damar No. 1 and Canada balsam as mounting media for histological sections. Nevillite V and No. 1 are superior to the natural resins in that they are homogeneous, clear, transparent, water-white, pure, neutral, have a higher melting point (which is desirable for micro-projection), a slightly higher refractive index, and are less expensive. The color of Nevillite V is more stable than that of Nevillite No. 1 and its refractive index is a little lower; otherwise they are of equal value. A 60% solution of the Nevillites in 40% toluene by weight is recommended.—*W. R. Hunt.*

DYES AND THEIR BIOLOGICAL USES

ALBERT, ADRIAN. **Acridine compounds in peace and war.** *Chem. and Ind.*, **60**, 253. 1941.

Some of the acridine derivatives are of great value in surgery because they kill disease germs without injuring human leucocytes. Of the common amino-acridines, namely acriflavine, euflavine and proflavine, the last has been most strongly recommended as an antiseptic by recent workers. It has been found that 2:7-diaminoacridine is a most promising antiseptic for application to the living brain in head injuries so common in war time. Because of the fluorescence of acridine compounds they are used in titrating opaque or highly colored solutions under an ultra-violet lamp. Radley and Grant include many acridine compounds in their list of standard indicators. Many other acridine drugs are used in human amoebiasis, malaria, tabes and lambliasis, as well as in mammitis, and other infections of cattle. Another spectacular property of certain acridine compounds is their ability to chemiluminesce or give off "cold light". Further development in this interesting research is necessary, especially on the methods of synthesis which are now tedious and unsuited to large scale manufacture.—*A. P. Bradshaw.*

FERREBEE, J. W., LEIGH, O. C. and BERLINER, R. W. **Passage of the blue dye T-1824 from the blood stream into the lymph.** *Proc. Soc. Exp. Biol. & Med.*, **46**, 549–53. 1941.

The injection of blue dye, T-1824, into the jugular vein of dogs was followed by the appearance of approximately 20 to 40% of the plasma concentration of dye in the thoracic lymph after 2 hr., and about 3 to 15% in the cervical lymph. It is estimated that about 7.5% of the injected dye passed thru the thoracic duct in 2 hr. The studies are pertinent to the knowledge of blood plasma volume in connection with shock.—*M. S. Marshall.*

ANIMAL MICROTECHNIC

BAKER, J. R. Chlorazol black E as a vital dye. *Nature*, **147**, 744. 1941.

Chlorazol black E (C.I. 581) possesses unusual ability to be taken up quickly and completely by the reticulo-endothelial system. A 1% solution in distilled water is convenient for subcutaneous injection into mice. The dye is non-toxic and is not excreted. Vitally stained preparations may be counterstained with safranin and orange G after Zenker fixation.—*S. H. Hutner.*

COPLEY, ALFRED L., and ROBB, TOM P. A new direct method of counting the blood platelets. *Amer. J. Physiol.*, **133**, 248. 1941.

One cc. of venous blood is drawn into a syringe containing 1 cc. of modified Aynaud solution (NaCl 0.75%, sodium citrate 3.8%, formaldehyde 3.7%). After mixing, the fluid is stored in a clean, dry, stoppered test tube. When a count is to be made the tube is inverted 10 times and 5 cc. of the mixture is added to 12 cc. of 3.8% sodium citrate. This second dilution is mixed and 0.5 cc. of it is added to 0.5 cc. of 0.2% brilliant cresyl blue in 3.8% sodium citrate. The final mixture is placed in a counting chamber. The color of the red blood cells fades after 10 min., and the platelets appear as dark blue bodies.—*Elbert C. Cole.*

DE BOISSEZON, P. Une modification de la technique de coloration de la myéline au noir Soudane B. *Bull. d'Histol. Appl.*, **18**, 90. 1941.

The technic described by Lison and Dagnelie (Bull. d'Histol. Appl., 12, no. 2, 1935) for staining myelin is unsatisfactory for study of the peripheral nervous system because of the dark preparations resulting from the dark color of the fat of the conjunctive tissue. The following modification has been developed to avoid this difficulty: Place the frozen sections in acetone for 15 min. (this dissolves the neutral fats leaving the myelin intact); 70% alcohol, 10 min.; Sudan black B dissolved in 70% alcohol and filtered, 5 min.; differentiate in 60% alcohol; wash in distilled water; mount in glycerin gelatin. This gives preparations in which the myelin nerves and their endings appear dark blue with a clear background.—*Jean E. Conn.*

GOMORI, G. Observations with differential stains on human islets of Langerhans. *Amer. J. Path.*, **17**, 395–406. 1941.

A study of the differential staining of α, β and δ cells of human pancreas (within 4 hr. after death) led to improvement of the chromium-hematoxylin-phloxine stain. The following procedure was used: Fix 2 mm. slices in Bouin's 30 min., then halve with a razor to 1 mm., and continue to fix for 24 hr. Prepare paraffin sections on slides and run down to water in the usual manner. Refix in Bouin's, 12–24 hr. Wash thoroly in tap water to remove picric acid. Treat 1 min. in an aqueous solution of 0.3% each of $KMnO_4$ and H_2SO_4. Decolorize with 2–5% aqueous sodium bisulfite and wash. Prepare the hematoxylin solution by mixing equal parts of a 1% aqueous solution of hematoxylin (National Aniline, C. P., or Grübler's dark) and 3% chromium alum; then, to each 100 cc. of the mixture, add 2 cc. of 5% $K_2Cr_2O_7$ and 2 cc. of 0.5 *N* H_2SO_4. Let ripen 48 hr. and filter. Stain under microscopic control until the β cells stand out deep blue (10–15 min.). Differentiate in 1% HCl-alcohol for about 1 min. Wash in tap water until the sections become a clear blue. Counterstain with a 0.5% aqueous solution of phloxine (source not stated) for 5 min. Rinse and transfer to 5% phosphotungstic acid solution for 1 min. Wash in tap water for 5 min., in which treatment the red color should reappear. Differentiate in 95% alcohol; but, if the section is too red and the α cells do not stand out clearly enough, rinse 15–20 sec. in 80% alcohol. Transfer to absolute alcohol, clear in xylene and mount in balsam. Sixteen figures which show the author's stain and also results with the Mallory-Heidenhain azan stain and Mallory's phosphotungstic acid hematoxylin are included, together with studies of the pancreatic islets under normal and pathological conditions.—*H. A. Davenport.*

LOWRY, OUIDA, BEAMS, F. H. W., and KING, R. L. **The fibrillae of the uterine cells of Ascaris equorum (variety univalens).** *J. Morphol. & Physiol.*, **68,** 585–91. 1941.

Pieces of the uteri of living worms were fixed in several fixing solutions, of which alcohol-acetic (9 parts of 70% alcohol and one part glacial acetic acid) was best. Tissues were embedded in paraffin and cut from 4 to 10 μ thick. Of several stains tried, Heidenhain's hematoxylin (following alcohol-acetic fixation) gave best results. Both basement membrane and fibers stained bluish black.—*Elbert C. Cole.*

MONKE, J. VICTOR. **A method for counting the leucocytes in blood containing gum acacia.** *J. Lab. & Clin. Med.*, **26,** 1664–7. 1941.

After the injection of gum acacia into the blood stream, it is not possible to obtain satisfactory leucocyte counts using Turck's Solution as the lytic diluent. The following dilution solution is recommended: Saponin, 0.1%, and methyl violet 0.1% in 0.85% saline. This solution produces complete hemolysis and stromolysis. The white blood cells are easily counted.—*John T. Myers.*

PAPANICOLAOU, GEORGE N. **Some improved methods for staining vaginal smears.** *J. Lab. & Clin. Med.*, **26,** 1200–5. 1941.

The following complete method is satisfactory for both human and animal vaginal smears: Fix the moist smear in a mixture of equal parts alcohol and ether, rinse in 70% alcohol, then 50% alcohol, then water. Place in Ehrlich's hematoxylin (or other hematoxylin) for 2 min. Rinse in distilled water, keep in running water for 5 min. or in Li_2CO_3 (3 drops concentrated aqueous solution in 100 cc.—exact concentration not stated) for 1 min. Rinse in distilled water. Stain in a counterstain such as the following for 2–5 min.: aniline blue W.S. (0.5% aqueous solution) 12 cc., orange G (0.5% aqueous solution) 25 cc., acid fuchsin (0.5% aqueous solution) 21 cc., eosin Y (0.5% aqueous solution) 42 cc., phosphotungstic acid 0.1125 g., phosphomolybdic acid 0.225 g. Rinse in water. Rinse in dioxan 10 to 15 times until clear. The staining time may be shortened by omitting the hematoxylin. Total cornified cells stain with the yellow dye. Smears must not be permitted to dry because the staining reaction of the cells will be changed.—*John T. Myers.*

TUREVICH, E. I. **Nucleal reaction of the elementary bodies.** *Bull. de Biologie et de Médicine Expérimentale de l' U.R.S.S.*, **9,** 222–4. 1940.

If nuclei stained violet by the Feulgen reaction are viewed by dark-field with carbon arc illumination, they show a bright yellowish-green luminescence (fluorescence ?); control nuclei not subjected to preliminary acid hydrolysis (*N* HCl for 6 min. at 60°C., then 1 min. in the cold solution), and which therefore do not stain, do not show this dark-field luminescence. The elementary bodies of infectious ectromelia, chicken pox and vaccinia, after treatment by the Feulgen procedure, are invisible by bright field, but display a green luminescence by dark-field similar to that observed with the nuclei stained for controls. The accompanying bacteria have a violet hue by bright field, and pronounced green luminescence by dark-field. It is concluded that the apparent negative Feulgen reaction of elementary bodies follows from the small size of the virus particles rather than from a lack of nuclear material.—*S. H. Hutner.*

PLANT MICROTECHNIC

JOHANSEN, D. A. **A quintuple stain combination and an evaluation of dyes suitable for mutiple staining.** *El Palo Alto News*, **6,** 1–4. 1941.

Exceptionally good differentiation of plant materials has been secured by the use of a combination of five dyes. Among the materials used to test the differentiation were mature embryos of corn, stems of *Aristolochia*, microsporocytes and megagametophytes of lily and thalli of *Nemalion*. Large numbers of slides may be stained in a rack by the following method: Pass the slides thru xylol down to 70% ethyl alcohol and then into a safranin solution (dissolve 4 g. safranin 0, NMv-6, in 200 cc. methyl cellosolve, add 100 cc. each 95% alcohol and distilled water, then add 4 g. sodium acetate and 8 cc. neutral formalin) for 24 hr. or more.

Rinse in running water 10 sec. Stain in a 1% solution of methyl violet NE (C.I. 680), in distilled water for 5 min. Rinse in running water 10 sec., then in equal parts methyl cellosolve, 95% ethyl or pure isopropyl alcohol, and tertiary butyl alcohol for 10 sec. Stain 5 min. in the green dye (dissolve completely 1 g. fast green FCF, LGf-1, in 200 cc. methyl cellosolve, add 1 g. malachite green, LMg-3, C.I. 657, shake thoroly, and, when dissolved, add 100 cc. each 95% ethyl alcohol and tertiary butyl alcohol, acidify with glacial acetic acid to make about 1%). Rinse in 0.5–0.8% glacial acetic acid solution in water for 10 sec. Rinse in cellosolve alcohol mixture as used in previous step acidified with glacial acetic acid to 0.5%, 10 sec. Stain about 5 min. in an orange solution (a saturated solution of orange I, C.I. 150, or orange II, C.I. 151, in methyl cellosolve; to this add 100 cc. each clove oil, 95% ethyl alcohol, and tertiary butyl alchol). Rinse 10 sec. in equal parts methyl cellosolve, absolute ethyl or isopropyl alcohol, tertiary butyl alcohol, clove oil, and terpineol (not terpinol). Rinse 10 sec. in equal parts xylol, absolute ethyl or isopropyl alcohol, methyl cellosolve, tertiary butyl alcohol, and terpineol. Rinse 10 sec. in xylol plus 10% absolute ethyl alcohol. Rinse 10 sec. in each of two changes of xylol. Mount in balsam dissolved in benzol.

Other dyes were suggested for various special purposes. The dyes were from National Aniline or Hartman-Leddon; and the technic has been standardized for the particular batches mentioned. This makes it a difficult procedure to follow; this is especially true since the designation NMv-6, given for Safranin 0, happens to be a certification number applying to a batch of methyl violet.—*Virgene Kavanagh.*

KISSER, J. **Versuche über Trockenkonservierung von Zweigen und Knospen in natürlicher Form und Farbe.** *Ber. deut. botan. Ges.*, **58**, 256–68. 1940.

The natural form in twigs and buds may be preserved by laying them in water until they have swollen completely, then transferring to 40–50% glycerin until thoroly saturated. If the material has been killed and fixed, it is especially important to get complete penetration of the glycerin solution. Since long soaking is harmful to the color and produces abnormal bendings of the buds, repeated evacuations of the immersed twigs with the filter pump, followed by leaving several days in the evacuated condition, are advisable. If the pieces are large or the buds impervious, it will be necessary to pierce the tissue with a fine needle. When infiltration is complete, the material is brought out of the solution, dried superficially and laid in an airy place until the water has evaporated.

The green color can be preserved by the use of copper salts (10% $CuSO_4$, 7% $Cu(CHO_2)_2$ or 5% $CuCl_2$) in 10% acetic acid. Should the resulting color be too bluish, a salt of zinc (1 part copper to 1 to 5 parts zinc) or of uranium (1 part uranium to 5–10 parts copper) may be substituted for part of the copper salt.

The brown color is seldom affected by the agents used in preserving the green. When disturbing colorations appear, a treatment with a solution of SO_2 is possible without damage.

The preservation of the red or anthocyanin colors is still not possible in many cases, but in twigs and buds it is best obtained by means of formol in the solution.

A fluid recommended for twigs and buds with exceptionally brown, or brown and red colors is 25 cc. formol and 75 cc. of 60% glycerin. Where there is only green, or green with red or brown, a mixture of 25 cc. formol, 10 cc. acetic acid, 50 cc. glycerin and 15 cc. H_2O is preferred. To the latter mixture should be added the desired amounts of powdered salts of copper or copper with zinc or uranium.—*Merritt N. Pope.*

SCHAEDE, R. **Über den Feinbau von Parenchymmembranen.** *Ber. deut. botan. Ges.*, **58**, 275–90. 1940.

For investigating the fine design structure of the parenchyma membrane in the pith and root tissue of various plants, the chlorzinciodide of Van Wisselingh was used. It is prepared by dissolving 15 g. $ZnCl_2$ in 10 cc. distilled water and adding 0.5 g. KI and 0.25 g. iodine. The iodine is in excess and insures a concentrated solution. After about 2 days at room temperature, the solution takes on an amber color and is ready for use, giving an intense, pure, dark blue stain. If the preparation is ringed, it will hold its color for about a month.—*Merritt N. Pope.*

SCHUSTER, C. E. **Method for softening filbert buds imbedded in paraffin.** *Botan. Gazette,* **102, 815–7. 1941.**

The following procedure, altho possibly unsuited for the study of fine details, is recommended for gross studies of pollen tubes, tube cysts, and similar developments in the pistil of hard buds like those of the filbert:

Killing and fixing fluid. Solution A: 2% aqueous chromic acid, 10 cc.; 95% ethyl alcohol, 100 cc.; 36% acetic acid, 100 cc. Solution B: 40% formalin, 40 cc.; 95% ethyl alcohol, 100 cc. Mix solutions A and B in equal parts.

Procedure. Dissect out those parts of the buds needed for study and immerse in this fluid for at least 24 hours. Boil in 50% dioxan in a water bath under a ventilating hood for 40 min. Pour off dioxan and run thru several baths of ZnCl in increasing strength, prepared as follows: Dissolve 10 g. ZnCl in 35% HCl, and dilute with distilled water, in the following volumetric proportions for each of the first four baths: 1:7; 1:3; 1:1; 3:1. The fifth bath is undiluted. Treat 2 hr. in each of the first three baths, 1 hr. in the fourth and 15–30 min. in the fifth according to the degree of hydrolysis desired. Then dehydrate in three changes of 25% dioxan, and one each of 50% and 75%, allowing 2 hr. in the first strength and 1 hr. in each of the others; then 3 half-hour changes of 100% dioxan mixed with equal parts of butyl alcohol, adding 1 drop of oil (kind unspecified) to each cubic centimeter of the last change.

Put in vials with shaved paraffin, place on top of paraffin oven overnight, and in oven later for 3 or 4 half-hour changes of pure paraffin. Do not keep in oven longer than necessary, to avoid hardening the hairs.

Cut embedded material into blocks ⅓-½ inch square and soak in water for several months (e.g. 6 months in the case of filbert buds). Place for 2 weeks or more in open dishes containing a bacterial infusion prepared by decaying straw and grass in 0.05% aqueous NH_4NO_3; keep in a warm room out of sunlight, and as evaporation progresses add more of the NH_4NO_3 solution. Neither of these two steps alone produce sufficient softening for good sectioning.

After the softening, whole buds can be sectioned at 10μ, the pistils and attached tissue at 5–7μ. During sectioning the paraffin blocks (previously chilled in an ice-bath) are cooled from time to time with pieces of ice. The knife should be specially sharpened according to the directions of Evenden and Schuster. —*K. A. Stiles.*

MICROÖRGANISMS

FIELD, J. W. **A new rapid method of staining malarial parasites in thick blood films.** *Bull. from Inst. Med. Research (Federated Malay States),* **No. 2** *of 1941.*

Solutions of methylene blue (containing also methylene azure) and of eosin, made isotonic with blood plasma, both buffered to pH 6.6, are used to provide a new rapid method for staining thick blood films. The use of isotonic solutions avoids the osmotic stresses to which Giemsa-stained thick films are exposed. The "finger-slide" and "pipette" methods are recommended for making the films, which may be stained within a few minutes of preparation (as soon as they cease to be obviously moist). Fixation is unnecessary. The method of staining is as follows: Dip the film for 1 or 2 sec. into stain A (methylene blue, 0.8 g.; azure I, 0.5 g.; anhydrous Na_2HPO_4, 5.0 g.; anhydrous KH_2PO_4, 6.25 g.; distilled water, 500 cc.). Rinse 5 sec. in clean water. Dip for 1 or 2 sec. into stain B (eosin, 1.0 g.; anhydrous Na_2HPO_4, 5.0 g.; anhydrous KH_2PO_4, 6.25 g.; distilled water, 500 cc.). Rinse for 2 or 3 sec. in clean water. The solutions are prepared by dissolving the phosphates first, then adding the stain, and filtering after 24 hr. (The writer used eosin from the British Drug Houses and the other two dyes from Gurr.) The slides are placed vertically to dry; the best blood picture is obtained at the lower edge of the film.

The advantages claimed for this method are shortening of the preliminary drying of the blood, reduction of the time of staining, better preservation of the form of leucocytes and blood protozoa, and maintenance of correct pH by buffering. The only serious drawback is that the stain is liable to give confusing appearances in anemic blood; the chromatoid and reticular residues of immature red cells stain more obstrusively than with Giemsa's stain and may confuse an inexperienced worker.—*Jean E. Conn.*

GRAY, P. H. H. Staining of bacteria and certain fungi. *Nature*, **147**, 329. 1941.

Spores and vegetative cells may be stained differentially in one operation by the following method: stain fixed films of bacteria or yeasts for 1 min. over steam with a solution of 0.5% malachite green and 0.05% basic fuchsin in distilled water; wash and dry. Spores stain greenish-blue, vegetative cells violet or pink. Acid fast bacteria retain the greenish-blue tinge, sometimes showing violet granules. A saline dilution of the staining solution has been developed for differentiating the hyphae and conidia of certain fungi in agar plate cultures. More details are to appear in a later publication.—*Jean E. Conn.*

HORVATH, J.v. Die Anwendung der Karminessigsäure für die Kernfärbung bei den Ziliaten. *Zts. wiss. Mikr.*, **57**, 168–71. 1940.

The author uses the aceto-carmine method for nuclear staining of ciliates of the Hypotrichae, Peritrichae, and Holotrichae. The stain is prepared as follows: glacial acetic acid 45 ml., distilled water 50 ml., add excess of powdered carmine and gently boil from ½ to 1 hr.; when cool, filter and make up to original volume; preserve in a glass-stoppered bottle. The routine technic is varied as follows:

(a) If material is abundant, use the centrifuge method (Gelei, 1934), employing equal quantities of culture and staining fluid and applying 15-20 min.; put on slide and place cover glass over it. For permanent preparations, add 10% glycerin to the staining fluid before centrifuging; and after decanting, replace the discarded fluid with more of the same glycerin-aceto-carmine solution before transferring to a slide.

(b) For individual specimens, spread the material evenly on albumenized slides, fix and stain with aceto-carmine. A small drop of glycerin and cover glass complete the preparation.

For many organisms (e.g. cultures of Peritrichae in straw infusion or in horse manure) special fixation may be necessary since acetic acid does not fix completely; in this case, 50% glacial acetic acid in absolute alcohol is used with the culture medium in a relationship of 1:1; fixation 5 to 15 min. After fixing the following procedure is recommended: Centrifugalize, pour off fixative, and without washing, add aceto-carmine, staining with occasional shaking 1 to 24 hr. After staining differentiate by placing the test tubes into a water bath from 60°–80° C. for 3–4 min. Agitate tubes frequently. If differentiation is insufficient, add 2–3 drops of glacial acetic acid per each 0.5 ml. stain (with material) and repeat differentiation in water bath at 80° C. If over-differentiation has taken place, pour off the old staining mixture and add a fresh mixture, allowing the material to stand for 5 min. at room temperature.—*J. M. Thuringer.*

PIEKARSKI, G. Über kernähnliche Structuren bei Bacillus mycoides Flügge. *Arch. Mikrob.*, **11**, 406–31. 1940.

Of 11 fixing agents tested with the Feulgen reaction, the best was concentrated $HgCl_2$ in water. Poor fixation resulted from the use of methyl alcohol, formalin and chromic acid. Consistently good results were obtained with *no* fixing agent. Staining was as follows: Hydrolyze in *N* HCl 8–10 min. (25–35 min. for spores) at 58°–60° C., rinse in distilled water and stain for at least 1½ hr. in fuchsin-sulphurous-acid (stain spores up to 24 hr.), place in an aqueous solution of SO_2 for 10 min. and rinse briefly in tap water. Use alizarin-viridin in alcoholic solution as a plasma counterstain.—*Merritt N. Pope.*

WEISS, EMIL. A modification of the Gram stain. *J. Lab. & Clin. Med.*, **26**, 1519–21. 1941.

The following technic is recommended: Prepare thin uniform smears. Cover with a solution of 3% gentian violet in 20% alcohol for 3–5 min. Wash with warm water. Cover for 3–5 min. with Gram's iodine of 20 times the usual concentration. Cover with acetone and wash off immediately with water. Counterstain briefly with fuchsin (2% of basic fuchsin in 95% alcohol). The technic is simple, the reagents keep well and the distinction between Gram negative and Gram positive bacteria is good.—*John T. Myers.*

HISTOCHEMISTRY

BARBER, H. N., and PRICE, J. R. **Nature of the Feulgen reaction with nucleic acid.** *Nature,* **146,** 335. 1940.

In checking Semmens' suggestion that the Feulgen reaction is due to purine components of nucleic acids, these authors find that the effect of the piperidine and pyridine is not chemically equivalent to the Feulgen (Schiff) reaction, but is simply due to their basicity, the effect of which was pointed out by Feulgen. Tests of three of the purines used by Semmens gave negative results. It is concluded that the Feulgen reaction is specific for the potential aldehyde groups of chromatin.—*M. W. Jennison.*

BARNETT, S. A., and BOURNE, G. **Use of silver nitrate for the histochemical demonstration of ascorbic acid.** *Nature,* **147,** 542–3. 1941.

The specificity of the test for ascorbic acid by reduction of an acid solution of silver nitrate may be increased by treatment of the specimen with 5% ammonia after the silver nitrate. Some tissues such as developing bone may show nonspecific deposition of silver otherwise.—*S. H. Hutner.*

SEMMENS, C. S. **Nature of the Feulgen reaction with nucleic acid.** *Nature,* **146,** 130–1. 1940.

This modified Schiff's reaction is commonly used as a selective chromatin stain. It is generally stated that the color reaction is due to the aldehyde group of the aldose in hydrolyzed nucleoprotein. The author found that such heterocyclic compounds as pyridine and piperidine also restored the original color of the fuchsin. Caffeine, theobromine, adenine and guanine gave positive results in a few hours. It is suggested that the Feulgen reaction with chromatin may therefore be due to the purine components of the nucleic acids.—*M. W. Jennison.*

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